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Models of Anti-Cancer Therapy

Human Tumor Xenografts as Predictive Preclinical Models for Anticancer Drug Activity in Humans

Better Than Commonly Perceived—But They Can Be Improved

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ABSTRACT

It is not uncommon for new anti-cancer drugs or therapies to show highly effective, and sometimes even spectacular anti-cancer treatment results using transplantable tumors in mice. These models frequently involve human tumor xenografts grown subcutaneously in immune deficient hosts such as athymic (nude) or severe combined immune deficient (SCID) mice. Unfortunately, such preclinical results are often followed by failure of the drug/therapy in clinical trials, or, if the drug is successful, it usually has only modest efficacy results, by comparison. Not surprisingly, this has provoked considerable skepticism about the value of using such preclinical models for early stage in vivo preclinical drug testing. As a result, a shift has occurred towards developing and using spontaneous mouse tumors arising in transgenic and/or knockout mice engineered to recapitulate various genetic alterations thought to be causative of specific types of respective human cancers. Alternatively, the opinion has been expressed of the need to refine and improve the human tumor xenograft models, e.g., by use of orthotopic transplantation and therefore promotion of metastatic spread of the resultant 'primary' tumors.

Close inspection of retrospective and prospective studies in the literature, however, reveals that human tumor xenografts—even non metastatic ectopic/subcutaneous 'primary' tumor transplants—can be remarkably predictive of cytotoxic chemotherapeutic drugs that have activity in humans, when the drugs are tested in mice using pharmacokinetically clinically equivalent or 'rational' drug doses. What may be at variance with clinical activity, however, is the magnitude of the benefit observed in mice, both in terms of the degree of tumor responses and overall survival. It is argued that this disparity can be significantly minimized by use of orthotopic transplant/metastatic tumor models in which treatment is initiated after the primary tumor has been removed and the distant metastases are well established and macroscopic—i.e., the bar is raised and treatment is undertaken on advanced, high volume, metastatic disease. In such circumstances, survival should be used as an endpoint; changes in tumor burden using surrogate markers or micro-imaging techniques can be used as well to monitor effects of therapies on tumor response. Adoption of such procedures would more accurately recapitulate the phase I/II/III clinical trial situation in which treatment is initiated on patients with advanced, high-volume metastatic disease.

INTRODUCTION

One of the greatest challenges faced by developers of new drugs and treatment strategies for cancer is the obvious need to test them in preclinical in vivo models that have a good probability of being predictive of similar activity in humans. For more than half a century the laboratory mouse has been the primary species in which experimental cancer treatments have been tested. Until about 25 years ago syngeneic transplantable mouse tumors were used most commonly for such preclinical therapy studies, and still are, especially for immunotherapy experiments in which an intact immune system is required. The discovery that human tumor cell lines, and sometimes even primary biopsy human tumor specimens, can give rise to progressively growing, and potentially lethal cancers in immune deficient mice gradually resulted in a shift towards the use of human tumor xenografts for the study of virtually all other types of anti-cancer drugs and treatment strategies.[1] Essentially every clinically approved anti-cancer drug was tested using these models, and showed positive anti-cancer effects before being evaluated in early, and then late phase clinical trials. Nevertheless, these successes have been overshadowed by highly visible failures in which a particular new anti-cancer drug, or treatment strategy, demonstrated remarkable

anti-tumor effects using a transplantable tumor model in mice, only to be followed by failure in the clinical trial setting[2] ("failure" in this case being defined here as having little or no survival benefit, regardless of whether it was found to be safe, or not, in humans).

Perhaps the most spectacular and recent example of this was the study by Boehm, O'Reilly et al.[3] who reported stunning effects of endostatin on three different transplantable tumors subcutaneously grown in syngeneic mice: the Lewis Lung carcinoma, the B16 melanoma and the T41 fibrosarcoma.[3] Cycles of daily endostatin treatment, an antiangiogenic protein drug, caused repeated and total regressions of established tumors. There was no evidence of relapse involving emergence of drug resistant variant/mutant subpopulations. Leaving aside the question of whether this result is reproducible (most other published studies of successful endostatin therapy show much more modest growth delays, but not overt tumor regressions), this result sparked enormous interest in both the scientific literature[4] and lay press.[5] It fueled unprecedented rapid initiation of phase I clinical trials in the United States, the results of which were recently reported.[6,7] The results of these trials showed the drug to be safe (which is the primary purpose of phase I trials) but there was certainly no evidence of the type of spectacular preclinical responses that had been observed in any of the treated patients.[6,7] The inevitable result has been the disappointment expressed not only about the drug itself, but about antiangiogenic therapy in general. In fairness, the results of other clinical trials involving antiangiogenic therapy such as the humanized monoclonal antibody to vascular endothelial cell growth factor (VEGF) known as bevacizumab (tradename: Avastin), which was tested in a randomized phase III trial as a third line therapy combined with Xeloda in advanced metastatic breast cancer, have also contributed significantly to this sense of current disappointment. But even in this case the disappointment stems, in part, from the many impressive results of prior preclinical studies utilizing a variety VEGF targeting of antiangiogenic drugs and approaches in a variety of mouse tumor models.

In 1999, Dr. Judah Folkman was quoted in a Newsweek magazine article as saying that a mouse study does not belong on the front page of the New York Times.[8] This makes considerable sense, and was a logical follow up to a quote he made in the May 3, 1998 Sunday New York Times article: "if you are a mouse and have cancer, we can take good care of you".[5] This statement would also seem to be logical, but as explained in this review, it is not necessarily so, and can be seriously challenged. Simply put, if you are a mouse with advanced, high-volume metastatic disease we probably cannot take good care of you.

The apparent lack of predictability of results often obtained using transplantable mouse or human tumors in normal or immune deficient mice has convinced many investigators to move away from such models and instead use spontaneously arising tumors, in particular genetically manipulated transgenic/knockout mice where the tumors which arise have mutations thought to be causative of the respective human cancers.[9,10] Alternatively, other investigators have suggested that transplantable tumor models can be made much more predictive by orthotopic transplantation which frequently facilitates metastatic spread—especially of human tumor xenografts[11,12]—and thus testing the effects of a given therapy on either (or both) the primary tumor growing in a physiologically relevant site (as opposed to an ectopic site) and distant metastatic disease.

In this commentary, two major points are made:

1. growth and testing of human tumors in subcutaneous tissue sites that are ectopic for a given type of cancer have provided relevant and predictive information to the clinic, provided that clinically relevant, pharmacokinetic parameters (especially dosing) are employed; and,
2. orthotopic transplants are nevertheless potentially valuable when used to generate metastases—but that therapy should be initiated at a point when the metastases are well established and macroscopic in nature (i.e., high volume metastatic disease)—and not just on low-volume (occult) minimum residual disease, which is what almost all previous studies have utilized when testing therapies on metastatic disease.

Also highlighted is the need for continuous vigilance with respect to the nature and origin of the cell lines used for transplantable tumor studies.

RETROSPECTIVE STUDIES OF CHEMOTHERAPEUTIC DRUGS USING SUBCUTANEOUS/ECTOPIC HUMAN TUMOR XENOGRAFTS SHOWING A HIGH DEGREE OF CLINICAL RELEVANCE

Nomura, Inaba and colleagues of the Cancer Chemotherapy Centre, Japanese Foundation for Cancer Research, Tokyo, have published a series of important and insightful studies which show clearly the remarkable potential of ectopic human tumor xenografts for predicting the pattern of activity of conventional cytotoxic chemotherapeutic drugs in humans.[13-17] Prior to undertaking their studies many other published reports showed that the majority of chemotherapeutic drugs have significant anti-tumor effects on a particular type of human cancer, even though most of the drugs tested were known not to have such activity on the respective tumor type in the clinical situation. In other words, the results of preclinical xenograft models were not retrospectively predictive of clinical activity. However, Nomura, Inaba and colleagues reasoned this could be due to inappropriate drug dosing. It turns out that the maximum tolerated dose (MTD) of most chemotherapeutic drugs that be given to mice is higher (4–5 times) than in humans. In some cases, the MTD is lower in mice than in humans, and in some cases (e.g., adriamycin) it is the same. Thus, in many cases, if one uses the MTD of a given chemotherapeutic drug for mice, the blood levels of drug will be significantly higher than can be attained in humans, leading to false positive tumor responses in mice.

To study this hypothesis, Nomura, Inaba and colleagues tested a large number of independent cell lines (e.g., generally eight to twelve) for each type of cancer tested. They reasoned this was similar in nature to the number of patients in a typical phase I clinical trial, and as such, would minimize the risks associated with obtaining a false positive or false negative response when testing just a single or few cell lines. In other words, one looks for an overall pattern of response in mice to different drugs that may be similar to what is seem a population of cancer patients. Each tumor cell line was grown as subcutaneous xenograft in a number of athymic nude mice. These mice were subsequently treated with at least 5 or 6 different chemotherapeutic drugs, tested as monotherapies, where some of the drugs were known to be clinically active on the particular type of human cancer being tested, and some not. The drugs were administered to some groups of tumor-bearing mice using the MTD of the drug for mice, whereas in another group the pharmacokinetically clinically equivalent dose (CED) or "rational dose" for humans was used.

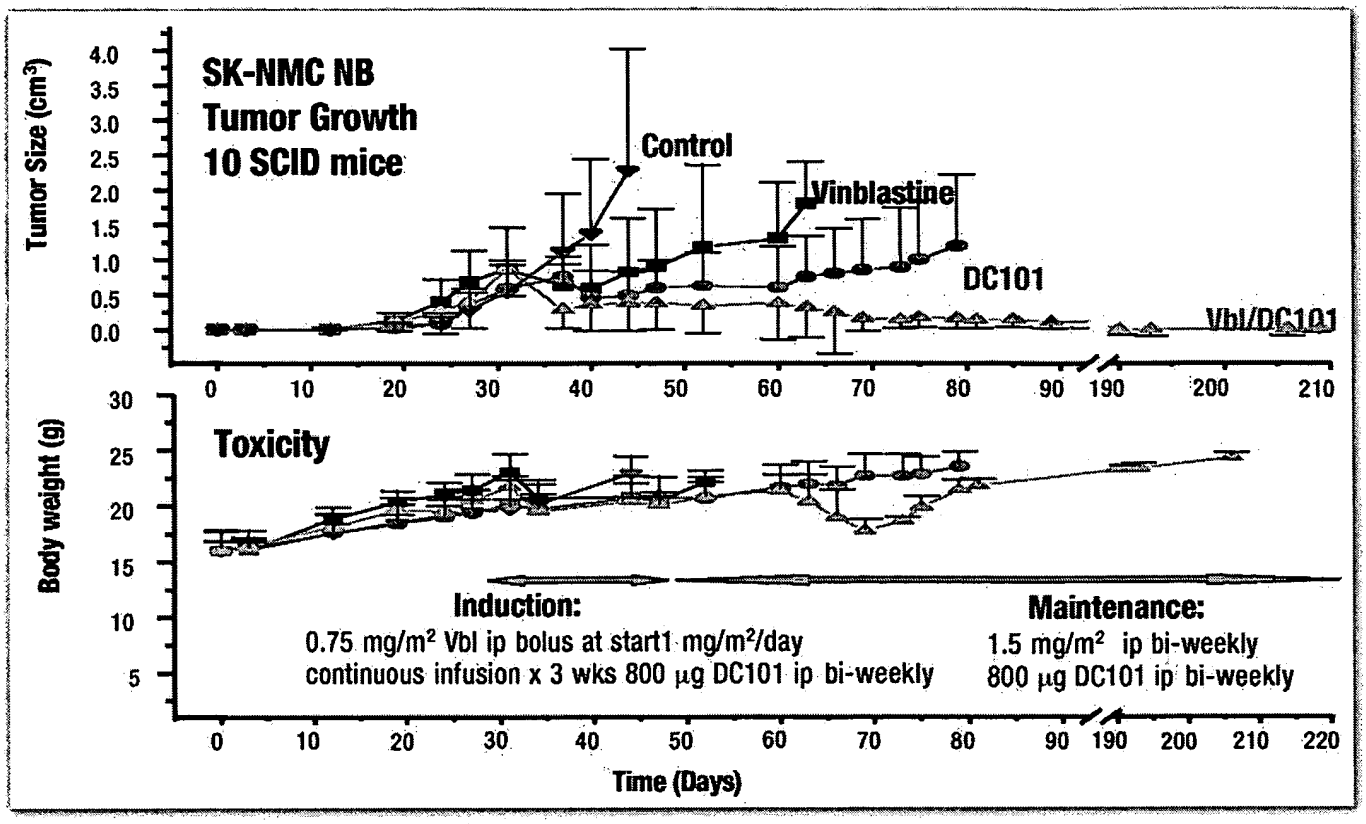

Figure 1. Results of an experiment in which large, established (0.75 cm^3) human neuroblastomas (NB) were treated with a metronomic low-dose vinblastine schedule, or DC101 (an anti-VEGFR-2 monoclonal antibody) or a combination of the two drugs. The dosing of the drugs is indicated in the lower figure. Note that the metronomic/ maintenance regimen was preceded by an induction regimen of the same drug to try and rapidly debulk the tumor mass before initiating the metronomic low-dose chemotherapy schedule. Progression of disease was seen in the single treatment groups, whereas slow but eventually complete tumor regression was noted in the combination group in which the therapy was continued for 7 months, which was possible by the lack of toxicity of this regimen. Taken from Klement, G. et al. "Continuous low-dose therapy with vinblastine and VEGF receptor-2 antibody induces sustained tumor regression without overt toxicity" *J Clin Invest* 2000; 105:R15-R24.

Analysis of the data for a large number of tumor types including lung, glioma, breast and gastric cancers showed that the pattern of response obtained when the mouse MTD was used was not associated with clinical pattern of responsiveness—most or all drugs showed activity. In other words, there was a high rate of false positives. In striking contrast, when the clinically equivalent or rational dose was used, the pattern of response in mice was similar to the activity of the respective drugs in the respective human cancer.[13-18]

These results were obtained using over 60 different established human cancer cell lines, all of which were injected subcutaneously. In no case was orthotopic injection of a cell line undertaken.

PROSPECTIVE STUDIES USING SUBCUTANEOUS HUMAN CHILDHOOD TUMOR XENOGRAFTS

Houghton and colleagues at St. Judes Children's Hospital in Memphis have also undertaken an exhaustive series of pharmacokinetic investigations in which a variety of pediatric malignancies were tested as subcutaneous xenografts in nude mice with respect to response to a variety of chemotherapeutic drugs. In particular, the relationship between systemic exposure and tumor response was evaluated, with emphasis on topoisomerase inhibitors such as irinotecan or topotecan.[19-24] These studies showed that a panel of neuroblastoma xenografts was highly sensitive to irinotecan, especially when administered using protracted schedules with lower doses of drug. For example, irinotecan was administered intravenously (i.v.) daily 5 days per week for 2 consecutive weeks (defined as one cycle) and compared to more protracted low-dose schedules where cycles were repeated every 21 days for a total of three courses. In the latter the total amount of drug was 5–10 mg/kg and was given using a daily schedule for 5 days, which was repeated 2 out of every 3 weeks for 9 weeks. Complete responses were observed in most of four of five xenografts using the intensive one cycle 40 mg/kg MTD schedule but the tumors tended to regrow. In contrast, with one exception, all neuroblastomas tested showed complete responses (CRs) which did not regrow during therapy when the protracted low-dose schedules were used involving a total dose of 10 mg/kg or 5 mg/kg.[23] Estimation of the lowest effective dose using the protracted i.v. schedule indicated that neuroblastomas respond to daily doses as low as 1.25 mg/kg.[23] It is interesting to consider these results in the light of those obtained by other investigators using a variety of similar protracted low-dose "metronomic" chemotherapy regimens as a putative antiangiogenic therapy, where increased efficacy and reduced toxicity have been frequently noted using such schedules, compared to the MTD of the same drug.[25-31]

The preclinical studies of Houghton and colleagues were directly translated to the clinic where the same protracted schedule was used and found to be well tolerated in children with refractory solid tumors; in addition encouraging, if not remarkable, rates of clinical responses were observed as well—16 of 23 patients experienced

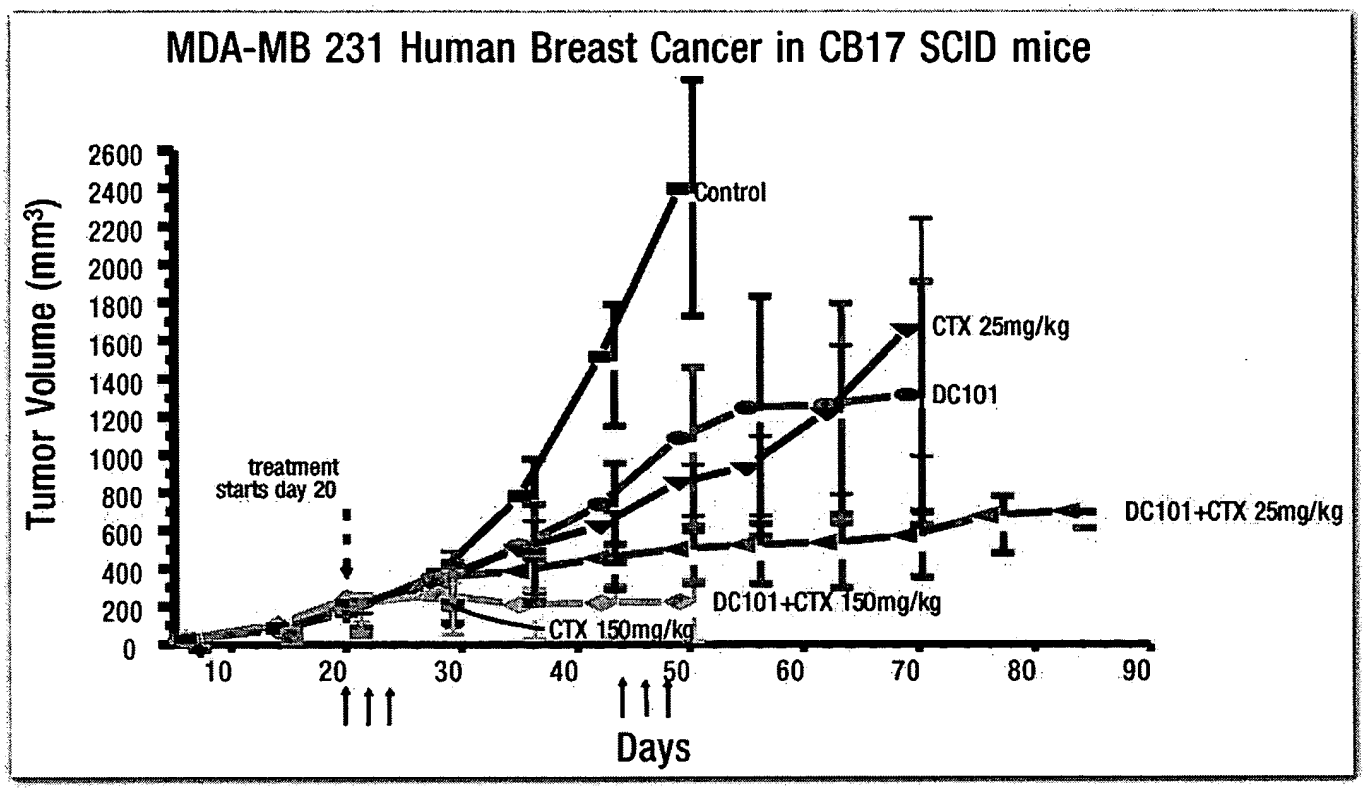

Figure 2. Results of an experiment published recently (Man et al. "Anti-tumor and anti-angiogenic effects in mice of low-dose (metronomic) cyclophosphamide administered continuously through the drinking water." Cancer Res., 62: 2731-2735, 2002) in which a human breast cancer cell line was injected "orthotopically" into the mammary fat pads of severe combined immunodeficient (SCID) mice, which allowed the tumor to metastasize to the lungs, liver and lymph nodes of the mice. Therapy was initiated when the "primary" intramammary fat pad tumor attained a size of 200 mm^3 and the disease had metastasized in a microscopic fashion only. Mice were then administered cyclophosphamide through their drinking water on a continuous non-stop basis at an estimated dose of 25 mg/kg per day, or treated with the DC101 anti-VEGFR-2 monoclonal antibody. In addition, another group of mice were given cyclophosphamide in the MTD fashion, i.e., at 150 mg/kg once every two days over a 6-day period (indicated by the vertical arrows). This MTD regimen was highly toxic to the SCID mice and resulted in death within one to two weeks. In contrast, mice given the same drug metronomically showed no signs of toxicity despite receiving up to 3 times the cumulative maximum tolerated dose given acutely.

stable disease and 5 showed partial responses.[24] These results show that preclinical xenograft models, even those involving ectopic/subcutaneous transplants, can provide useful predictors of the activity and responses of some pediatric cancers to topoisomerase I inhibitors such as irinotecan. A more detailed overview and discussion of the testing of new agents in childhood cancer models, both xenografts and transgenic oncomouse models was recently published by Houghton et al.[32]

IMPROVING HUMAN TUMOR XENOGRAFT MODELS FOR PREDICTING THE RELATIVE BENEFIT OF ANTI-CANCER DRUGS IN HUMANS—THE IMPORTANCE OF TREATING (ADVANCED) METASTATIC DISEASE

While the results summarized above are encouraging, and clearly show the potentially predictive value of human tumor xenografts, there is an aspect of the results in many of the preclinical studies that is nevertheless troubling: the excellent, if not remarkable, nature of the tumor responses in mice, as such responses are infrequently observed in cancer patients even though the drug being tested may be active against its respective human counterpart. For example, as discussed above, Houghton et al. observed complete responses of established solid neuroblastoma xenografts in a high proportion of cases using various irinotecan dosing schedules, especially protracted low-dose protocols.[23] However, such dramatic responses were not observed in the respective clinical trial of 23 patients, which included five children with neuroblastoma.[24] It is this aspect of experimental therapy studies in mice that can be frustrating as it often attracts considerable attention (e.g., the endostatin studies of Boehm, O'Reilly et al. discussed above) and expectation. This disparity has caused considerable skepticism about what to expect in the clinic on the basis of prior preclinical therapy studies. However, this skepticism may not always be justified when one takes into account, in retrospect, a crucial and fundamental difference between virtually all published experimental mouse therapy studies and corresponding clinical trials, and it is this: in most phase I, II and III clinical trials the patients being treated have advanced, high-volume metastatic disease whereas most mouse studies do not test the effects of therapy on advanced metastatic disease, but rather on a primary tumor transplant or spontaneously arising primary tumor, or microscopic, low-volume metastatic disease (Lee Ellis, personal communication). With respect to treatment of metastatic disease, typically, in such experiments, tumor cells are injected intravenously to generate lung or liver tumor colonies ("artificial metastases"), and therapy is initiated within one or a few days after injection of the cells—if not before tumor cell injection! This constitutes a form of adjuvant (or prophylactic) therapy, on microscopic, low-volume metastatic disease. Alternatively, growing primary tumors may be surgically removed, and treatment then initiated within a few days when the spontaneous metastases that have formed are microscopic in size. Thus, there is a much less demanding therapeutic situation for mice than for humans, when it

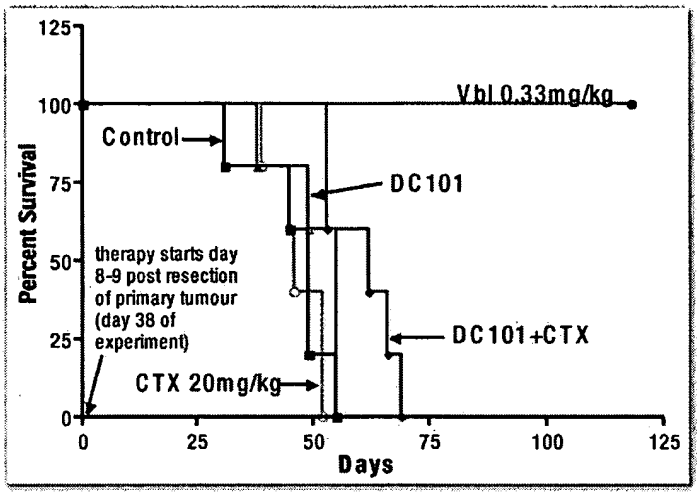

Figure 3. Effect of various therapy regimens on survival of SCID mice with advanced, metastatic cancer at the time of initiation of therapy. SCID mice were inoculated with MD-MBA-435 human tumor cells. The inoculation was into the mammary fat pads which facilitated distant metastatic spread, provided the primary tumors are surgically excised. This was done approximately 4 weeks after tumor cell inoculation, after which therapy was initiated approximately 8-9 days later. The cyclophosphamide was given continuously through the drinking water at an estimated dose of 20 mg/kg per day, whereas vinblastine or taxol at the indicated dose were injected at low-dose twice a week.

comes to comparing most preclinical trials to the clinical trial counterparts. Perhaps much of the disparity in results between the two is related to this variable since it is well known that high-volume advanced metastatic disease is generally much more difficult to treat than low-volume adjuvant disease. Add to this the fact that many patients entered into clinical trials had been treated previously with other therapies and have relapsed with refractory disease. Heavily pretreated and resistant patients are often less responsive to a new therapy, and usually have advanced metastatic disease at the time of entry into a clinical trial.[33] How often have investigators in the past tested a new drug or therapy in mice where this dire clinical situation is recapitulated? The answer is rarely—if ever.

To illustrate the point about treating (advanced) metastatic disease, some recent results obtained in this laboratory are shown. Figure 1 shows the results of an experiment in which a metronomic low-dose vinblastine protocol, in combination with an antiangiogenic drug, called DC101 (an anti-VEGF receptor-2 blocking antibody) was used to treat large, established human neuroblastoma xenografts obtained after subcutaneous injection of SK-NM-C cells.[27] The results showed a remarkable anti-tumor effect could be obtained with the combination—sustained and complete tumor regressions. In effect, the mice were cured since the therapy was continuously maintained for 7 months,[27] and surprisingly, tumors did not resume growth when the treatment was finally terminated (unpublished observations). However, because the tumors were injected subcutaneously (i.e., ectopically) they did not metastasize, and therefore the much more demanding clinical situation of treating advanced metastatic neuroblastoma metastases was not duplicated in the mouse studies. The preclinical study was not intended to predict clinical activity—as implied by a headline proclaimed on the front page of a prominent national Canadian newspaper,[34] but to illustrate the principle of metronomic low-dose chemotherapy as a relatively non-toxic and effective way of giving chemotherapy, and combining it with a targeted antiangiogenic drug.[27,35-38]

Figure 2 shows the results of a similar experiment in which a human breast cancer (MDA-MB-231) was injected orthotopically in the mammary fat pads of female SCID mice, and then treated continuously with an oral low-dose regimen of cyclophosphamide administered continuously through the drinking water, combined with the same antiangiogenic drug, DC101.[29] A control using an MTD regimen of cyclophosphamide was also used. In terms of survival, the best treatment regimen was the combination of the metronomic oral low-dose cyclophosphamide/ DC101, and the survival benefit was obvious. However, in this model, while the orthotopic breast cancer can metastasize, the metastases remain largely microscopic because of the retention of the primary tumor and the timing of the initiation of treatment. Thus, treatment of low-volume, metastatic disease was undertaken.

More recent experiments have involved 'raising the therapeutic bar', so to speak. In Figure 3 a tumor cell line, called MDA-MB-435, supposedly a well known breast cancer cell line used extensively in breast cancer research, was injected into the mammary fat pads of SCID mice and allowed to grow for about one month. The resultant primary tumors were then surgically removed and initiation of treatment with oral low-dose cyclophosphamide and/or DC101 was delayed for about 10 days to allow establishment of extensive macroscopic metastases in the lungs and draining lymph nodes of the SCID mice, as well as diffuse metastatic spread in the liver (data not shown). Using survival as an endpoint, neither DC101 alone or oral low-dose cyclophosphamide alone had had impact on survival; the combination did have an effect, but the magnitude of the benefit was rather modest in comparison to the sort of results shown in Figures 1 and 2. Of considerable interest, however, was the finding that a metronomic low-dose vinblastine protocol—0.33 mg/kg given intraperitoneally three times a week—alone caused complete resolution of advanced metastatic disease and greatly prolonged survival of the mice. Eventually, the mice had to be sacrificed because tumors recurred at the site of surgical removal and grew progressively in spite of the success of the therapy on distant metastatic disease (unpublished observations).

It is of course difficult to compare the results of each experiment since different tumor cell lines and different treatment regimens were used. Indeed, the MDA-MB-435 'breast' tumor cell line has recently been implicated to be a melanoma, based on gene and protein expression profiling,[39,40] results which we have confirmed using the MDA-MB-435 line discussed in Figure 3. Nevertheless, the results of Figure 3 do suggest that treatment of advanced metastatic disease in mice will give results that may turn out to be much more reflective, i.e., predictive, of the clinical situation typically encountered when testing new drugs in phase I, II or III clinical trials. The vinblastine therapy results also point to the possibility that we cannot always assume that the response of a primary tumor will mirror the effects of the same therapy on distant metastases—this is obvious. What is not so obvious, and surprising, is that the response of metastases may be significantly better than the primary tumor in some cases. We would anticipate that this would be the exception rather than the rule; nevertheless this has ramifications for anti-cancer screening and drug testing, if correct.

CONCLUSIONS

In light of these results one might want to rethink Dr. Folkman's quote "if you are a mouse and have cancer, we can take good care of you".[5] One may argue this applies to mice with rapidly growing, transplanted, subcutaneous, encapulated/non-metastatic tumors. In contrast, mice with high-volume, advanced, metastatic disease in sites such as the lungs, liver and brain may not be so easy to take care of, similar to their human counterparts. The vinblastine results do however provide some basis for optimism, and emphasize the need to begin testing models which involve advanced metastatic disease. This, incidentally, is one of the limitations of many of the current transgenic oncomouse models, as they usually do not spontaneously metastasize.[41,42] Moreover, monitoring the effects of therapies on metastatic disease in mice is becoming easier and less subjective with the growing use of small animal non-invasive micro-imaging research tools[43] and non-invasive biochemical techniques, e.g., measuring secreted tumor-specific protein markers that can be introduced into tumor cell lines.[29,44] It is also time to reexamine some of the current dogmas regarding mouse models of cancer. First, human tumor xenografts can be surprisingly predictive of clinical activity, and in some cases this includes subcutaneous/ectopic transplants. The wisdom of the rush towards exclusive use of much more expensive transgenic oncomouse models for drug therapy testing can be questioned, especially when such tumors fail to express the most critical element of malignant disease—ability to metastasize, and the fact that less expensive transplantable tumor models are available which work—if used appropriately.

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